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(54) Title: CONJUGATES AND CONSTRUCTS INCLUDING ANTI-CD28 AND ANTI-CD3 BINDING MOLECULES

(57) Abstract

Disclosed are substances useful for proliferating T cells and inhibiting tumor growth, comprising a polymer backbone or microbead adsorbed to or conjugated with binding molecules which preferably target the CD3 or CD28 antigen on T cells, or other T cell antigens, and which is administered *in vivo*. The binding molecule may be a whole antibody or a fragment thereof which preferably lacks an Fc portion, and in either case should not induce immunosuppression or T cell depletion. Specific examples include a latex macrosphere conjugated with, or a polylactide microsphere adsorbed to, an anti-CD28 antibody of $F(ab')_2$ fragment thereof and/or an $F(ab')_2$ fragment of an anti-CD3 antibody.

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Conjugates and Constructs Including Anti-CD28 and Anti-CD3 Binding Molecules

Field of Invention

The invention relates to conjugates and constructs including anti-CD28 or anti-CD3 binding molecules and polymers, or other backbones, for inducing specifically the polyclonal activation, proliferation, and/or lymphokine production of T lymphocytes, or subsets thereof, in order to enhance the immune system's ability to fight tumors and infections.

Background of the Invention

Most immune responses involve many components of the immune system. Although the immune mechanisms involved in the elimination of malignantly transformed cells are not well understood, it is reasonable to assume that if more immune mechanisms are activated and enhanced, the tumorous cells may be eliminated more effectively. Also, both humoral and cellular mechanisms are known to be involved in the immune response against viruses and virus-infected cells. Thus, generally speaking, for treatment of patients with various cancers or infectious diseases, and for protecting individuals exposed to infectious agents from contracting the infection, it is desirable to enhance the entire immune system.

The various branches of the immune system include antibodies, cytotoxic T cells (CTLs), T cells mediating delayed-type hypersensitivities (T_{TDH} cells), monocytes and macrophages, natural killer (NK) cells, killer

cells mediating ADCC, and granulocytes. Complex interactions are involved in the activation of these various branches. The helper T cells (T_h cells) play central regulatory roles, and many factors are secreted by these cells and other cells in a certain concerted fashion during the activation and proliferation phases. There is good reason to believe that the concerted production of lymphokines and cytokines, at the appropriate time and in the proper relative proportions, is important for maximizing the immune response.

Potentiation of the immune system is desirable for treating a number of pathological conditions, *e.g.*, for treatment of malignant tumors, such as those associated with renal cell carcinoma and malignant melanoma. The immune potentiators include substances identified from screening natural sources, such as cultures of microorganisms, marine animals, herbs, or plants, as well as substances screened from large batteries of synthetic organic compounds.

One example of a substance from a natural source is muramyl dipeptide, which has been identified as the smallest structure from the cell wall of staphylococcal bacteria which still retains immune potentiating effects. Many analogues of muramyl dipeptide have been synthesized. Muramyl dipeptide and its analogues are macrophage activators, and have been tested and developed as therapeutic agents for tumors and as adjuvants for vaccines.

Other examples of immune potentiators derived from natural sources include double-stranded RNA and mismatched double-stranded RNA (also called ampligen) which can induce interferon synthesis and other immune functions. These substances have also been tested for treating tumors and viral diseases, such as AIDS.

Immune potentiators may be applied to patients alone or in combination with surgery, irradiation, or chemotherapy. They may also be desirable for treating patients with viral infectious diseases or for protecting individuals, after exposure to viruses, from contracting infection. Immune potentiators may be useful as adjuvants for various vaccines for infectious diseases or cancers.

Recently, recombinant human lymphokines and cytokines have been produced by genetic engineering. Many such recombinant "biological response modifiers" are being tested for treatment of various cancers and infectious diseases. A few recombinant products, such as interleukin-2 (IL-2), α-interferon, γ-interferon, granulocyte-colony stimulation factor and granulocyte/monocyte-colony stimulation factor (G-CSF, GM-CSF), have been approved in many countries for use against certain cancers and infectious diseases. For example, IL-2 is approved for treating patients with renal cell carcinoma; α-interferon is approved for treating patients with hairy cell carcinoma or with hepatitis B infection; G-CSF and GM-CSF are approved for treating cancer patients receiving chemotherapy for the

purposes of restoring lost neutrophils.

Individual recombinant lymphokines, such as IL-2, IL-4, or γ-interferon can augment some aspects of the immune system, but function only against limited immunocyte targets and can only potentiate certain immune functions and not the entire immune system. They also probably function only over short ranges and in limited areas *in vivo*. Also, cytokines and lymphokines which are injected into patients are cleared rapidly through the kidneys. They likely will not be present in sufficiently high concentrations in the lymphoid system for long enough to achieve their desired immunological effects.

Of the various substances other than lymphokines or cytokines which have been studied for potentiating the immune system, most which are suitable for *in vivo* use do not target or enhance the T cells directly. For example, muramyl dipeptide, and analogues thereof, primarily activate macrophages. Double-stranded RNA and mismatched double-stranded RNA mainly induce interferon production by a variety of cells.

A few naturally-derived protein substances are known to be potent T cell mitogens in culture *in vitro*, and have been used in studies to characterize and quantitate T cell activity. These substances include phytohemagglutinin A (PHA), concanavalin A (Con A), wheat germ agglutinin (WGA), and some other lectins, defined as carbohydrate-binding plant proteins. However, these T-cell mitogenic proteins, although very

useful for *in vitro* studies, have poor specificity and therefore bind to almost all cell types. Because they are toxic and lack specificity, they are not effective for *in vivo* use as T cell potentiators.

In order to activate and expand lymphocytes to achieve satisfactory therapeutic effects while avoiding administering toxic substances, some groups have sought to activate and expand the T lymphocytes from patients in culture *in vitro* for a period of time under optimal conditions and then harvest the activated cells and inject them back into the same patients. In this so-called IL-2/LAK therapeutic regimen, used by the Biological Therapy Institute (Franklin, Tennessee) to treat patients with various cancers, the blood is first drawn from the patients and the mononuclear cells are isolated. *See* Rosenberg, S.A. et al., *N. Eng. J. Med.* 316:889 (1987). The cells are incubated in medium containing recombinant IL-2 for several weeks, and the activated and expanded T cells, which contain the lymphokine-activated killer (LAK) cells, are harvested and injected into the patients.

A more recent, modified version of this IL-2/LAK therapy, known as autolymphocyte therapy (ALT) has been developed by Cellcor Therapies, Inc. in Boston Massachussetts. *See* Osband, M.E. et al., *Lancet* 335:994 (1990). The lymphocytes from renal cell carcinoma patients are taken twice. The first time, the lymphocytes are stimulated with antibodies specific for human CD3 antigen (anti-CD3) *in vitro* to produce

lymphokines. The culture supernatant is collected after a few days of culturing, and the cells are discarded. The second time, the lymphocytes taken from the patients are incubated in the autologous lymphokines for a period of five days and the cells are harvested and injected into the same patients.

It is claimed that these approaches, involving *in vitro* lymphocyte stimulation and expansion, achieve beneficial responses in a portion of the treated patients. The major concern with these regimes is that the treatment is very tedious, expensive, and requires sophisticated, specialized cell culture facility. The variation among cells or cultures from different patients requires demanding monitoring procedures. Also, lymphocyte cultures have very poor viability even under optimal conditions, meaning that during the culturing, large numbers of the cells will die. When large numbers of dead cells are injected into patients, this may actually burden the reticuloendothelial system (RES) and reduce its effectiveness in combating the tumor cells.

In summary, the clinical studies and approved routine uses of IL-2 and γ-interferon and of LAK or ALT therapies indicate that T cell activation and expansion can achieve therapeutic effects in some patients with cancers or infectious diseases. On the other hand, the results of these treatments suggest that the lymphokine treatments have certain deficiencies and the LAK and ALT treatments have some substantial drawbacks. Thus,

an efficacious and feasible treatment may be realized if these deficiencies can be eliminated.

A number of MAbs specific for CD3 on the surface of human T cells (pan T marker) are known to be very potent mitogens of human T cells *in vitro*, *e.g.*, the MAb OKT3. Van Wauwe, J.P. et al., *J. Immunology* 124:2708 (1980); Chang, T.W. et al., *Proc. Natl. Acad. Sci. U.S.A.* 78:1805 (1981); MAb 64.1 Hansen, J.A. et al., *Leukocyte Typing: Human Leukocyte Differentiation Antigens Detected by Monoclonal Antibodies,* Eds. Bernard, A. et al. (Spring Verlag, New York, 1984). In medium containing only fetal calf serum and no human serum (and therefore no IgG), the anti-CD3 MAbs are much more potent than PHA or Con A in inducing T cell proliferation.

But the mitogenic effect of anti-CD3 requires both specific binding to the CD3 antigen and the presence of the Fc moiety of the antibody, as well as the presence of monocytes and macrophages. The best explanation for these results is that the Fc of the anti-CD3 MAbs binds to the Fc receptors on monocytes/macrophages, thereby aggregating the CD3 antigen on the T cell surface. Since CD3 is associated with the T cell antigen receptors, the aggregation of CD3 triggers the activation and proliferation of the T cells.

This explanation is supported by experiments which show that when the anti-human CD3 MAb is conjugated to Sepharose 4B beads or coated

on the substratum plastic surface of culture wells, monocytes and macrophages are not needed to induce activation and proliferation of T cells. *See* Williams, J.M. et al., *J. Immunol.* 135:2249 (1985); Ceuppens, J.L. & Baroja, M.L., *J. Immunol.* 137:1816 (1986); Geppert, T.D. & Lipsky P.E., *J. Immunol.* 138:1660 (1987). Based on these experiments, it has been suggested that the solid-phase anti-CD3 MAb functions by aggregating the CD3 antigen on the T cell surface.

However, when anti-human CD3 is injected *in vivo*, the results are the opposite of the *in vitro* effects. OKT3 MAb, which is the first MAb ever approved for therapeutic use *in vivo*, is strongly immunosuppressive and is approved for use as an immunosuppressor for patients receiving kidney transplants. Ortho Multicenter Group Study, *N. Eng. J. Med.* 313:337 (1985). The injection of OKT3 causes rapid depletion of T cells from the circulation. Although the mechanism by which anti-CD3 causes this rapid depletion of T cells is not well understood, the best explanation is that anti-CD3 induces ADCC of the T cells, *i.e.*, as the T cells coated by anti-CD3 circulate through the spleen and liver, they are lysed by the phagocytic cells of the RES in these organs. It is also possible that some of the T cells are destroyed by complement-mediated cytolysis and some other cytolytic mechanisms.

In *in vivo* mouse studies using a hamster MAb against murine CD3, it has been shown that low doses of anti-CD3 can prevent malignant

progressive tumor growth and protect against lethal sendei virus infection. Ellenhorn, J.D. et al., *Science* 242:569 (1988); Kast, W.M. et al., *J. Immunol.* 145:2254 (1990). It has been suggested that the T cells in the mice are activated by such treatment with anti-CD3. Hirsch, R. et al., *J. Immunol.* 142:737 (1989).

Human and murine studies involving *in vivo* administration of anti-CD3 indicate that there is a substantial difference between the two species. In humans, even minute amounts of anti-CD3 are immunosuppressive and cytolytic. Also, the activation and mitogenic effect of anti-CD3 on T cells is completely blocked by the presence of human serum or IgG. Chang, T.W. et al., *Proc. Natl. Acad. Sci. U.S.A.* 78:1805 (1981); Looney, R.F. and Abraham, G.N. *J. Immunol.* 133:154 (1984). These results suggest that whole anti-CD3, or fragments thereof, will not activate T cells *in vivo*, and therefore, the invention described below is not suggested.

Summary of the Invention

The immunoregulatory substances of the invention include binding molecules which target the CD3 or CD28, or other antigens on T cells, and which preferably lack an Fc portion or have the Fc portion inactivated, conjugated with or adsorbed to a polymeric backbone (for example, polylactide, polyethylene glycol ("PEG")), latex, polylactide, cellulose, dextran, agarose, or an amino acid copolymer), a liposome, a long chain peptide (preferably containing Lys or Cys residues, more preferably,

containing Gly, Ser, and Lys (or Cys) at 20:4:1 ratio, with molecular weights of 10,000 to 1,000,000 (about 150 to 15,000 amino acid residues in length)), or a microbead. The binding molecules can be, for example, Fv, Fab, or $F(ab')_2$ fragments, or whole antibodies. The conjugation can be covalent or non-covalent. The other antigens on T cells which can be targeted include CD2, CD4, CD5, CD8, and CD37.

The main use for the immunoregulatory substances is as immune potentiators in humans or animals, which activate and expand T cells or a subset of the T cells, and stimulate them to produce IL-2, γ-interferon, IL-1, IL-4, IL-6, tumor necrosis factor (TNF), or other lymphokines. Because T cells play central roles in the regulation of many branches of the immune system, the concerted secretion of a number of lymphokines will activate many immune mechanisms, whereas the administration of individual lymphokines may have a more limited effect.

The immunoregulatory substances of the invention may be used to treat patients or animals with cancers or infectious diseases, or to protect individuals exposed to infectious agents from contracting the infections. The immunoregulatory substances may also be used as adjuvants for vaccines, which could reduce the number of times that a vaccine needs to be administered in order to be effective in prophylaxis. This could be particularly effective for vaccination against diphtheria, influenza, and measles, as there already are mass vaccination programs for children

against these diseases.

The immune potentiators could also be used in veterinary practice, particularly to treat companion animals affected with cancers or chronic infections. For use in veterinary practice, the same substances of the invention mentioned above are employed, with the fragments and antibodies targeting the T cell antigen of the animal one is seeking to treat. Among the diseases in companion animals which might be particularly well suited for treatment with the products of the invention are the canine distemper adenovirus, coronavirus, or Rabies virus, and the feline leukemia virus. Embodiments of the invention suitable for treating feline leukemia virus include antibodies and fragments which target feline CD3, which are coupled with microbeads or other polymer backbones.

The substances of the invention can also be used diagnostically, in standard assay formats such as an ELISA, to detect cells bearing the CD3 and/or the CD28 antigen. This can be useful to determine the number of cells in an unknown fluid sample which bear these antigens.

Detailed Description of Making and Using the Invention

The Fv fragments of the MAbs may be produced in bacteria using single chain antibody technology, as described in U.S. Patent No. 4,946,778 and International Application No. WO88/09344. The Fv may also be genetically engineered to contain glycosylation sites and produced in mammalian cells, to result in a fragment containing carbohydrate

moieties.

The Fab or $F(ab')_2$ may be produced by enzymatic cleavage of whole IgG, which can be produced by a hybridoma or a transfected cell lines (a myeloma or a cell line such as Chinese Hamster Ovary (CHO)), using pepsin or papain digestion, respectively.

The Fab or $F(ab')_2$ fragments, or the whole antibodies, may be wholly animal or human derived, or they may be in chimeric form, such that the constant domains are derived from the constant regions of human immunoglobulins and the variable regions are derived from the parent murine MAb. Alternatively, the Fv, Fab, or $F(ab')_2$ may be humanized, so that only the complementarity determining regions (CDR) are derived from an animal MAb, and the constant domains and the framework regions of the variable regions are of human origin. *See, e.g.*, U.S. Patent Application Serial No. 07/952,802, filed 9/25/92, for a detailed example of how to make a particular type of humanized antibody. These chimeric and humanized antibodies and fragments are less immunogenic than their wholly animal counterparts, and thus more suitable for *in vivo* use, especially where administration will be over prolonged periods.

Methods of making chimeric and humanized antibodies are well known in the art, (*see, e.g.*, U.S. Patent No. 4,816,567, International Application No. WO84/03712, respectively). The Fv, Fab, or $F(ab')_2$ fragments may be produced from such chimeric or humanized antibodies

using proteolytic digestion, as described above.

The antibody fragments can be conjugated to the linear or cross-linked backbone, polypeptide, microbead or liposome using conventional techniques, well known in the art. *See, e.g.,* Ostro, M.J. (Ed.), *Liposomes: from Biophysics to Therapeutics* (Marcel Dekker, New York, 1987). One preferred method of preparing liposomes and conjugating immunoglobulins to their surface is described by Ishimoto, Y. et al., *J. Immunol. Met.* 75, 351-360 (1984). Multilamillar liposomes composed of dipalmitoylphosphatidylcholine, cholesterol and phosphotidylethanolamine are prepared. Purified fragments can then be coupled to the phosphatidylethanolamine by the cross-linking agent N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate. The coupling of the antibody or fragment to the liposome can be demonstrated by the release of a pre-trapped marker, *e.g.,* carboxyfluorescence, from the liposomes upon the treatment of secondary antibody against the conjugated antibody, fragment and complement.

The antibodies or fragments may also be coupled to a liposome or another carrier of the invention via their carbohydrate moieties. Provided that the carbohydrate moiety is not in the hypervariable region or at the antibody binding sites, the conjugation via the cross-linking with the carbohydrate will not affect binding, as the binding sites will still be available to bind to cell surface antigens.

One preferred way to couple antibodies and fragments of the invention (other than Fv) to a polymer backbone or a liposome is to conjugate them through the carbohydrate moiety on the constant regions. This will maximize the binding sites which are available, and not hindered, for binding to the antigens.

Methods for derivatizing sugar ring moities to create hydrazide groups for coupling with fragments (and antibodies) have been established. *See* Rodwell, J.D. et al., *Proc. Nat'l Acad. Sci. USA* 83:2632-36 (1986). Several immunoconjugates prepared in this way are in clinical studies or pending approval for routine clinical uses.

The polymers for conjugating to the antigen binding sites can be modified to generate active groups for coupling according to established methods. For example, PEG can be derivatized by 1,1'-carbonyldiamidazole to form imidazole carbamate active groups, which react with amino groups of proteins. Beauchamp, C.O. et al., *Anal. Biochem.* 131:25 (1983). Similar reactions can be used for derivatizing agarose. Bethell, G.S. et al., *J. Biol. Chem.* 254:2572 (1979).

The antibodies or fragments can be coupled directly to the derivatized, activated polymers. Bifunctional cross-linkers suitable for conjugating the activated polymers (or liposomes) and the antibodies or fragments, can be selected based on the properties desired and the specific substances to be cross-linked. These heterobifunctional reagents are

available from several commercial sources, *e.g.*, Pierce Chemical Co., Rockford, IL., and the reaction procedures are well-known.

The substances of the invention, in appropriate pharmaceutical vehicles, may be administered intravenously (i.v.), so that they can reach spleen, liver, and various lymph nodes. They will also reach the T cells in circulation when administered i.v.

The substances of the invention may also be given intraperitoneally (i.p.), where they will mainly interact with cells in the peritoneal cavity and will be delivered to other lymphoid tissues through the lymphoid circulation. The T cells which are activated and expanded in the spleen and peritoneal cavity may also travel to different tissues in the circulation.

The substances of the invention may also be injected directly into or near the solid tumors, warts, or other affected tissues. In this case, the T cells will be activated and expanded and mediate various immune mechanisms.

Certain substances of the invention may only induce the activation of resting lymphocytes and not their proliferation. In such case, their administration may be followed by T cell growth factors, such as IL-2 or IL-4.

The substances of the invention may be given alone, or in combination with surgery, irradiation treatment, or chemotherapy for cancer patients, or in combination with viral antibiotics or other anti-viral

substances for patients with infectious diseases.

Even without the animal model experiments described in the examples below, there is adequate experimental support for the efficacy of the invention. As noted above, the interaction between the Fc of the anti-CD3 MAbs and the Fc receptors (FcR) on monocytes/macrophages is required both for the mitogenic effect *in vitro* and the ADCC effect *in vivo*. It was first found that the $F(ab')_2$ and Fab fragments of OKT3, which lacked Fc, were no longer mitogenic and also that whole OKT3 could not induce the T cells to proliferate if the monocytes were depleted from the mononuclear cells in culture. Van Wauwe, J.P., et al., *J. Immunol.* 124:2708 (1980); Chang, T.W., et al., *Proc. Natl. Acad. Sci. U.S.A.* 78:1805 (1981). It was then suggested that the mechanism of anti-CD3 mitogenic effect involved the interaction between the Fc of anti-CD3 and FcR on monocytes, because human serum, purified human IgG, or isolated Fc fragments could block the mitogenic effect of OKT3. Looney, R.F. and Abraham, G.N., *J. Immunol* 133:154 (1984); Chang, T.W., *Immunol. Today* (1985). Several studies also showed that the deficiency of FcR for IgG on monocytes would impair the mitogenic effects of anti-CD3, supporting the notion that Fc-FcR interaction is important for the mitogenic effects of anti-CD3. Ceuppens, J.L., et al., *J. Immunol.* 135:3882 (1982); Tax, W.J.M., et al., *Nature* 304:445 (1983); Tax, W.J.M., *J. Immunol.* 133:1185 (1984).

A few studies examined the mitogenic mechanism of anti-CD3 further. It was found that anti-human CD3 MAb densely conjugated to sepharose 4B beads and purified human IL-1 could induce the proliferation of T cells in culture in which the antigen presenting monocytes were completely depleted. It was concluded that the anti-human CD3 MAb-Sepharose 4B could activate the resting human T cells and the IL-1 was then able to initiate the synthesis of RNA, IL-2 secretion, IL-2 receptor expression, and ultimately, DNA synthesis. Williams, J.M., et al., *J. Immunol.* 135:2249 (1985). It was also reported that in the complete absence of monocytes, the T cells could be induced to proliferate by incubation in plastic culture plates coated with anti-human CD3 MAb, if soluble anti-human CD5 MAb was also provided in the culture medium. Ceuppens, J.L. and Baroja, M.L., *J. Immunol.* 137:1816 (1986). Later it was reported that the resting T cells depleted of accessory monocytes could proliferate in wells of microtiter plates coated with high concentration of anti-human CD3 (for 64.1 MAb, 40-1000 ng/ml) without the addition of IL-2 or monocytes. Geppert, T.D. and Lipsky, P.K., *J. Immunol.* 138:1660 (1987).

Anti-CD3 and/or anti-CD28 MAb in an appropriate pharmaceutical composition, such as in bound or suspended form, may also be mitogenic instead of being immunosuppressive *in vivo*. However, solid plastic sheets or large Sepharose 4B beads, although suitable for *in vitro* use, may not be

appropriate for *in vivo* use. These solid materials, whether administered i.p. or by other routes, will be maintained *in situ*. They cannot be transported throughout the lymphoid system by the lymphoid circulation. For *in vivo* use, therefore, hydrophilic, soluble polypeptides, polymers, microbeads, or liposomes suitable for conjugating with large numbers of anti-CD3 or anti-CD28 MAbs are preferred.

When anti-CD3 or anti-CD28 MAbs are conjugated to the polymer backbones, microbeads or liposomes, the Fc portions will be accessible to monocytes and macrophages and other cells of the RES, and hence may facilitate phagocytosis and clearance of such conjugates. To minimize such clearance and to ensure that the mitogenic effect of anti-CD3 and anti-CD28 will be the dominant effect, and that any suppressive effect mediated by ADCC and complement-mediated cytolysis will be the lessened to low levels, fragments of anti-CD3 and anti-CD28 MAbs which are devoid of the Fc domains (*e.g.* Fv, Fab, and $F(ab')_2$) and which do not cause the Fc-dependent ADCC and complement-mediated cytolysis, may be conjugated to the polymer backbones, microbeads or liposomes. The experiments with solid-phase bound anti-CD3 MAbs suggest that under certain conditions, the Fc domain of the antibody is not required in mitogenesis.

Among the surface molecules that are involved in the regulation of the activities of lymphocytes, the most important seem to be the components or molecules associated with the TCR and CD28 on T cells. The TCR

complex is very complicated and the structure is not fully characterized, despite extensive study. The information available indicates that the "complete" TCR complex contains one α chain, one β chain, one γ chain, one ϵ chain, one ζ chain, and a homodimer δ chain. The α and β chains are clonally different and the α/β dimer is customarily referred to as the TCR. The remaining components of the TCR complex (γ, ϵ, ζ, and δ chains) are not polymorphic and are categorically referred to as the CD3 antigen.

A means to achieve cross-linking of antigenic epitopes on T cell surfaces is to use polymer backbones or microbeads coated or conjugated with anti-CD3 and anti-CD28 MAbs. To avoid the cytotoxicity caused by the Fc domain of the MAbs, $F(ab')_2$ derived from whole IgG, or genetically engineering $F(ab')_2$, are preferred. The conjugates may be implanted or deposited or administered i.v. into certain body sites in order to trigger mitogenesis. Thus, the appropriate backbone or base upon which to conjugate the MAbs should be polymers which are hydrophilic, stable, non-immunogenic, nontoxic and resistant to hydrolases (*e.g.* glycosidases and proteases) in the serum and other body fluids in patients. Examples are PEG, cellulose, dextran, polylactide, poly (DL-lactide), and latex beads which are glutaraldehyde modified, and agarose, which each have different molecular sizes and are all well-characterized and studied.

Another suitable "backbone" is an amino acid copolymer. Preferred

amino acid copolymers include Gly and Ser residues, and Lys, Cys, or other appropriate residues, for providing conjugation sites. Considering the molecular sizes of Fv, Fab, $F(ab')_2$, and whole antibodies, the optimal spacing between the adjacent Lys or Cys residues is in the range of 15 to 25 amino acids. Thus, a preferred amino acid copolymer has a composition of $(Gly_{15}Ser_5Lys)_n$, where n is 5 to 600.

The fragments or antibodies of the invention can also be conjugated to liposomes, using the methods described above, wherein reactive groups for cross-linking are introduced on the surface of the liposomes and the fragments are coupled thereto. For certain clinical applications with certain MAbs, fragments (or binding molecules) conjugated to liposomes may be more preferred than fragment/polymer conjugates, as the liposome conjugates can interact with antigen on T cells by a mechanism more closely resembling the interaction between cells, than when the fragment is presented on a polymer backbone.

Many polymers which are suitable as the backbone are available commercially in different lengths or sizes. Amino acid copolymers of different lengths can also be synthesized and fractionated by molecular sieve chromatography. Polymers such as cellulose or agarose can be treated with specific enzymes, *e.g.*, cellulase and agarase, to yield different lengths.

Good cross-linking and aggregation can also be obtained with other embodiments. For example, a polymer backbone can be coupled with a

number of monovalent Fab fragment against an antigenic epitope of a T cell. This embodiment functions similarly in principle to the other embodiments, the difference being that there are more binding sites in such molecular conjugates than in the $F(ab')_2$. Because of the larger numbers of binding sites, the cross-linking and aggregation will be more complete and there will be fewer singly-paired molecules, which are not cross-linked.

It is noted that, unlike the fragments of the invention which are mixtures of fragments binding to at least two different antigenic determinants, single MAbs which bind to monovalent antigenic determinants cannot cross-link the antigens on the cell surface. In order to stimulate cell activation and proliferation, cross-linking of the surface antigens is usually required. However, many surface molecules such as CD4 or CD8 are single polypeptide chains or are composed of different polypeptide chains, and cannot be efficiently cross-linked by a single divalent antibody recognizing monovalent antigenic epitopes.

The fragments of the invention, which are likely to have certain immunoregulatory effects in polymerized forms *in vivo,* include those which are specific for CD4, CD28, CD3 and components of TCR complexes, as well as other T cell antigens such as CD2, CD5, CD8, and CD37. Specific examples are fragments of the monoclonal antibody OKT3, which targets the CD3 antigen on T cells. Fragments binding to a surface antigen which is expressed by only T cells, or a subset of them, are potentially useful as

in vivo immunomodulators in polymerized forms.

Example 1: Developing Murine Monoclonal Antibodies Against the CD3 Antigen of a Non- Human Animal

A method of making monoclonal antibodies specific for the CD3 antigen of any mammal (including companion animals, such as dogs, cats, and horses, agricultural animals, such as cattle, and others) can be readily formulated from what is known about human and murine CD3 antigen and about the properties of antibodies specific for these antigens. The procedure is similar to those described for the making of anti-human CD3 monoclonal antibodies, by Kung, P.C. *et al* Science 206:347-349 (1979) and by Leo, O. *et al* Proc. Natl. Acad. Sci. U.S.A. 84:1374-1378 (1987).

The molecular weight and the subunit composition of the CD3 antigen is known for humans and mice, and is expected to be almost identical among different animal species. The human and murine sequences of CD3 subunit polypeptides are also known.

For immunizing mice against the CD3 antigen, fresh thymus tissue is obtained from the animal against which murine anti-CD3 monoclonal antibodies are to be prepared. Single cell suspensions are prepared by mincing the thymus tissue. The single cells are used for immunizing mice at dosage of 1×10^7 thymocytes per intraperitoneal injection per mouse, for a total of three injections at two week intervals between injections. Four days after the last injection, the mice are sacrificed and their spleens are removed for fusion with NS0 or SP/20 cells, following the standard

procedure for making hybridomas. The supernatants of the growing hybrids from the primary fusion wells in the microculture plates are screened for binding to the single thymocytes using immunofluorescence flow cytometry. The fluorescent probe for the immunofluorescent staining is FITC-labelled rat monoclonal antibody against murine IgG2a and IgG2b. Alternatively, the antibodies may be biotin-conjugated and used in combination with FITC-labelled avidin.

The fusion wells showing binding to the thymocytes are then cloned by limiting dilution, and the subclones are tested again for binding to the thymocytes using the immunofluoresence flow cytometry. The positive clones are further tested for mitogenic effects on T cells. For this test, peripheral blood is obtained by venipuncture. The mononuclear fraction, which contains lymphocytes and monocytes, is prepared by centrifugation on a Ficoll-Hypaque cushion, with the same procedure applied to human and murine mononuclear cells. A proliferative response assay employing ^{3}H-thymidine incorporation technique is used to determine the cellular proliferation. The clones with supernatants which are highly mitogenic on the mononuclear cells are then isolated for further characterization.

The specific reactivity of the monoclonal antibodies with the CD3 antigen can then be confirmed by, for example, the immunoprecipitation and Western blotting procedures. In the immunoprecipitation method, the thymocytes are surface-labelled with ^{125}I with a lactoperoxidase procedure.

Lysates of the labelled cells are prepared with NP40 detergent. The antigens reactive with the antibodies in question are bound and precipitated by the antibodies. The precipitates are then resolved by 1-D or 2-D SDS polyacrylamide electrophoresis (SDS PAGE). The pattern and the sizes of the bands are then compared with the CD3 antigen of human and murine origin, to confirm that the CD3 antigen, in fact, is bound.

In the Western immunoblotting method, the lysates of unlabelled thymocytes are run on SDS PAGE. The proteins are then electro-transferred onto a nitrocellulose paper, which is then incubated with the antibodies in question. After washing, the bound murine antibodies are localized with enzyme-conjugated goat IgG against murine IgG. The size of the reactive bands are compared to those of the human and murine CD3 antigen. For confirmation, the antigenic peptide can be isolated by 2-D SDS gel electrophoresis, and the N-terminal amino acid sequences can be determined and compared to those of human and murine CD3 antigen.

The appropriate antibodies can then be derivatized, and/or conjugated, as described above, in order to make products of the invention. These products can be used for T cell mitogenesis in non-human mammals.

Example 2: Testing Anti-CD3 MAbs for Noncompetitive Binding to CD3 on T cells

Various anti-CD3 MAbs can be purchased from commercial firms offering immunochemical reagents, including Ortho Diagnostic Systems,

Raritan, N.J.; Becton Dickenson Immunological Reagents, Mountain View, CA; Coulter Diagnostics, Hialeach, FL; Sigma Chemical Co., St. Louis, MO; Boehringer Mannheim, Indianapolis, IN; Olympus Corp., Lake Success, N.Y. All these MAbs were developed by different groups. These firms offer anti-CD3 MAb not only in purified, plain IgG, but also in fluorescein-conjugated forms.

Additional MAbs against human CD3 can be readily prepared by hybridoma methodology as described by Kung, P.C. et al., *Science* 206:347 (1979). Using this method, many laboratories have developed murine anti-human CD3 MAb. The same methods could be used to develop anti-CD3 MAb against the CD3 of virtually any mammal or companion animal. These techniques are well known in the art, and involve the standard techniques of immunization, fusion and screening used to make hybridomas and monoclonal antibodies.

For determining whether two MAbs (or fragments) specific for CD3 can bind to CD3-bearing T cells simultaneously, fluorescence flow cytometric analyses may be applied. For these analyses a T cell line, such as human CEM (ATCC CCL119 from the American Type Culture Collection), or peripheral blood mononuclear cells, can be used for the cell staining. The assay is to determine whether the binding of a FITC or rhodamine-labeled anti-CD3 MAb to the cells will be inhibited by the presence of varying concentrations of a second anti-CD3 MAb. The assay

should also be reversed to determine whether the binding of the fluorescence-labeled second anti-CD3 is inhibited by the presence of the other anti-CD3.

If the binding of each anti-CD3 to the T cells is not signficantly affected by 5-10 fold concentrations of the other anti-CD3, it can be concluded that both anti-CD3 MAb can bind non-competitively to CD3 molecules on T cells. Additional confirming assays would measure whether the binding to T cells by the two MAbs is additive.

Example 3: Conjugates of the Invention as T Cell Proliferation Enhancers in in vitro and Animal Model Systems

Conjugates of the invention were made by conjugating $F(ab')_2$ fragments of the hamster monoclonal antibody 145-2C11 (a gift from J. Bluestone), which is specific for murine CD3-ϵ chain, and the whole hamster monoclonal antibody 37.51 (purchased from PharMingen), which is specific for murine CD28, onto latex and polylactide microbeads. The latex beads (of a uniform 2.5 μm diameter), which were glutaraldehyde modified, were purchased from Interfacial Dynamics Corp. The latex beads were already modified to contain activated groups for coupling with proteins, and were also purchased from Interfacial Dynamics Corp. Suspensions of these beads could be made homogeneous and suitable for injection with gentle shaking. 0.4 to 0.7 μg of 145-2C11.$F(ab')_2$, and 4 μg of 37.51, were conjugated onto 1 mg of both the latex and the polylactide microspheres (also of a uniform 2.5 μm diameter).

An experment was run to determine cell proliferation on administration of the above product, as measured by [^{3}H] thymidine incorporation. Mouse spleen cells were suspended in RPMI-1640 medium supplemented with 2 mM glutamine and 5% fetal calf serum, containing a construct of the invention or a control (as described below). The suspension was plated in round bottom wells of microculture plates (96 well), and incubated for 48 hours in 5% CO_2 at 37°C. The cells were then pulsed with the [^{3}H] thymidine for 16 hours (0.5 μCi/well) and harvested on a semi-automatic harvester (Scatron Inc., Serling VA). The incorporation of ^{3}H into DNA in the cells was determined by scintillation counting.

The controls for the experiment were the antibodies 37.51, 145-2C11, the antibody 37.51 conjugated with 2.5 μm latex or polylactide beads, the F(ab')$_2$ fragment of the antibody 2C11 conjugated with 2.5 μm latex or polylactide beads, and the polylactide beads alone. The results with the controls were compared with the results achieved by conjugates of the latex or polylactide beads with the F(ab')$_2$ fragment of the antibody 2C11 and the antibody 37.51. The conjugation to the latex beads was done covalently, and the conjugation to the polylactide beads was done by adsorption (the polylactide beads are hydrophobic).

For the binding molecules covalently conjugated to the latex beads, the results were as follows:

145-2C11.F(ab')$_2$/latex beads: 70,000 cpm

soluble whole 145-2C11: 120,000 cpm

soluble 37.51: 3,000 cpm

37.51/latex beads: 25,000 cpm

37.51/145-2C11.F(ab')$_2$/latex beads: 135,000 cpm.

For the binding molecules non-covalently conjugated to the polylactide beads, the results were as follows:

145-2C11.F(ab')$_2$/polylactide beads: 85,000 cpm

soluble whole 145-2C11: 125,000 cpm

polylactide beads alone: 3,000 cpm

37.51/polylactide beads: 60,000 cpm

37.51/145-2C11.F(ab')$_2$/polylactide beads: 149,000 cpm.

It can be seen that the conjugate of the anti-CD28 antibody and the F(ab')$_2$ fragment of 145-2C11, or the conjugate of the F(ab')$_2$ fragment with a bead, significantly enhanced cell proliferation over that observed with the controls.

Example 4: T Cell Proliferation with Conjugates of the Invention

Further experiments were also ran to determine if F(ab')$_2$ fragments of 37.51 coupled to poly (DL-lactide) microspheres could induce T cell proliferation *in vitro*. A ^{3}H thymidine incorporation assay was used to determine T cell proliferation. Soluble whole IgG or its F(ab')$_2$ fragment of anti-mouse CD28 monoclonal antibody did not induce any T cell

proliferation. ^{3}Thymidine incorporation by the cells in culture remained at the background level, of about 2000-3000 cpm. However, when the $F(ab')_2$ fragment was adsorbed to poly (DL-lactide) microspheres, the ^3H thymidine incorporation by the T cells rose to about 25,000 cpm, indicating T cell proliferation.

Example 5: Conjugates of the Invention in a Mouse Tumor Model as Tumor Inhibitors

Using P815 mastocytoma and DBA/2 mice, the efficacy of the 37.51/145-2C11.$F(ab')_2$/polylactide bead conjugate in inhibiting this tumor was determined. The protocol was that on day 0, 13 DBA/2 mice were injected with 5×10^5 P815 cells. On day 7, all the mice developed tumors. 10 mice with tumor nodules of 5 mm or more were selected for the further studies, and these 10 mice were injected with the 37.51/145-2C11.$F(ab')_2$/polylactide bead conjugate at 8 μg per mouse (1/2 volume i.v., 1/2/volume i.p.). On day 14, injections with the conjugate were repeated. Beginning on day 21, the results were that only 5 of the treated mice bore tumors, and 5 of the treated mice did not.

In a separate control experiment, it was determined that untreated mice with the same tumor only survived for 35 days. Many of the treated mice survived for 45 days. Thus, it can be seen that this conjugate is effective in the animal model in inhibiting tumors.

The invention is not limited to conjugates of anti-CD3 fragments and anti-CD28 antibodies, but also includes all other anti-T cell antigen binding

molecules, fragments (and conjugates thereof) which are specific for surface antigens of human T lymphocytes, and which have immunoregulatory activities *in vivo*, when administered according to the techniques of the invention. Other binding molecules which target the T cell surface antigens, for example anti-CD4 and those specific for HLA class-1 antigens, HLA class-II antigens (such as Ia), CD2, CD5, CD8, or CD37, could also be included in conjugates, either separately or in combination with conjugates which include anti-CD3 and anti-CD28 binding molecules. Some of these antigens, such as CD2, CD4, CD5, and CD8 are specifically expressed by T cells or subsets of T cells.

It should be understood that the terms and expressions described herein are exemplary only and not limiting, and that the scope of the invention is defined only in the claims which follow, and includes all equivalents of the subject matter of those claims.

What is Claimed Is:

1. A molecular conjugate comprising a latex bead or support conjugated with binding molecules which target antigens on T cells.

2. The molecular conjugate of claim 1 wherein the binding molecules lack Fc portions.

3. The molecular conjugate of claim 2 wherein the binding molecules are $F(ab')_2$ fragments.

4. The molecular conjugate of claim 1 wherein the antigens are CD2, CD3, CD4, CD5, CD8, CD28 or CD37.

5. A construct comprising polylactide bead to which binding molecules which target antigens on T cells is adsorbed.

6. The construct of claim 5 wherein the binding molecules lack Fc portions.

7. The construct of claim 6 wherein the binding molecules are $F(ab')_2$ fragments.

8. The construct of claim 5 wherein the antigens are CD2, CD3, CD4, CD5, CD8, CD28 or CD37.

9. A polylactide microbead to which whole anti-CD28 antibody and $F(ab')_2$ fragments of an anti-CD3 antibody are adsorbed.

10. A polylactide microbead to which $F(ab')_2$ fragments of an anti-CD3 antibody are adsorbed.

11. A polylactide microbead to which $F(ab')_2$ fragments of an anti-CD28

antibody are adsorbed.

12. Latex microbeads conjugated with whole anti-CD28 antibody and $F(ab')_2$ fragments of anti-CD3 antibody.

13. Latex microbeads conjugated with $F(ab')_2$ fragments of anti-CD3 antibody.

14. Latex microbeads conjugated with $F(ab')_2$ fragments of anti-CD28 antibody.

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 35/00, 35/16; C07K 15/28, 17/00

US CL :424/85.8, 450; 530/388.75, 389.6, 387.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8, 450; 530/388.75, 389.6, 387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP A1 0,440,373 A1 (LEDBETTER) 07 August 1991, see entire document.	1-14
Y	WO A1 90/05541 (THOMPSON, et al.) 31 May 1990, see entire document.	1-14
Y	J. Immunology, Vol. 135, No. 4, issued October 1985, Williams et al., "The events of primary T cell activation can be staged by use of sepharose-bound anti-T3 (64.1) monoclonal antibody and purified interleukin 1," pages 2249-2255, see entire document.	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Immunology, Vol. 138, No. 6, issued March 15, 1987, Geppert et al. "Accessory cell independent proliferation of human T4 cells stimulated by immobilized monoclonal antibodies to CD3," pages 1660-1666, see entire document.	1-14